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Complement activation in human serum by liposome-encapsulated hemoglobin: the role of natural anti-phospholipid antibodies

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Abstract

In exploring the occurrence and mechanism of liposome-encapsulated hemoglobin (LEH)-induced complement (C) activation, we found that normal human serum contained low titers of IgG and IgM class natural antibodies with reactivity against LEH, and that the amount of vesicle-bound IgM significantly correlated with LEH-induced C consumption. IgM binding to LEH was inhibited by phosphocholine and ATP, but not by choline chloride. These data suggest that naturally occurring antibodies play a key role in LEH-induced C activation, and that a major portion of these antibodies are directed against the phosphate moiety on the phospholipid headgroups of liposome bilayers.

Keywords: Liposome; Complement; Hemoglobin; Natural anti-phospholipid antibody; Antibody

Liposome-encapsulated hemoglobin (LEH) represents a potential oxygen carrier blood substitute with a unique capability to imitate the hemoglobin (Hb) O_2-transport function of erythrocytes without risk for immune hemolytic reactions due to mismatched transfusion [1–4]. While the efficacy of LEH to provide life support has been demonstrated in exsanguinated rats [1,5], freedom from acute immune effects may not be complete. LEH has been shown to cause complement (C) activation in rats [6,7], which effect was suggested to underlie the transient cardiovascular, hematological and respiratory changes reported in this species following intravenous injection of this blood substitute [8–11].

In extending these studies to humans, we have found that incubation of LEH with normal human serum also induces C activation, as indicated by C consumption with associated elevation of C split products: C5a, SC5b-9, C4d and Bb [12]. We obtained indications for the involvement of both the classical (CP) and alternative pathways (AP) in LEH-induced C activation [12], however, that information cannot be regarded as conclusive with regard to the possible role of antibodies in the activation chain, as there are examples for CP activation of C through antibody-independent [13–16], as well as AP activation by antibody-mediated [17,18] processes.

An earlier study from our laboratory presented evidence that naturally occurring anti-cholesterol antibodies played a key role in the liposome-induced severe anaphylactoid reaction of pigs [19]. Yet, despite detailed investigations on the interaction of liposomes with plasma proteins [20,21] and extensive literature on liposome-induced C activation [13–

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16,22–25], the role of natural antibodies in liposome-induced C activation has not gained wide awareness to-date.

In the present study we have focused therefore on the role of antibodies in LEH-induced C activation. In particular, we wished to explore the presence of natural antibodies in human serum with reactivity to LEH, the class of these antibodies, their titer, specificity and role in LEH-induced C activation.

The preparation of LEH from distearoylphosphatidylcholine, dimyristoylphosphatidylglycerol, cholesterol and α-tocopherol (50:4.5:45:0.5 mole ratio, obtained from Avanti Polar Lipids, Alabaster, AL), using a microfluidizer, was described earlier [2,6,11,12,26]. For the present study we used a small unilamellar, highly homodisperse preparation, which was obtained by contract from Vestar (San Dimas, CA, now called Nexstar) and was prepared as described earlier [12]. In brief, human serum albumin (HSA) was coencapsulated with heat-sterilized, bis(3,5-dibromosalicyl)fumarate (diaspirin)-cross-linked human Hb (αα Hb) [27], the homogenization step was performed in a Gaulin homogenizer, unencapsulated protein was removed by tangential flow filtration, and the final product, containing 20 mg/ml αα Hb and 6 mg/ml HSA, was subjected to filtration through 0.45 μm pore size polycarbonate filters. Dynamic light scattering showed the mean vesicle diameter to be 186 ± 82 nm with narrow unimodal size distribution (i.e. 100% in main peak). Control liposomes containing HSA were prepared identically, except that αα Hb was omitted.

Details of the solid-phase ELISA developed to detect lipid specific antibodies were described earlier [28,29]. In brief, wells of 96-well Costar plates were coated with LEH (25 nmol phospholipid/50 μl PBS), blocked with 100 μl of 0.3% gelatin in PBS, washed with PBS and incubated with 50 μl serum at various dilutions for 4 h at room temperature. Plates were washed with PBS and the amount of LEH-bound antibody was measured using affinity-purified, horseradish peroxidase-conjugated anti-human IgG (γ-chain) or anti-human IgM (μ-chain) antibodies (Binding Site, Birmingham, UK) at 500-fold dilution. Values were corrected for background, i.e., A_{405} readings obtained in uncoated wells.

Binding of naturally occurring IgM to LEH in the presence of phosphocholine chloride, choline chloride and ATP (the latter compounds obtained from Sigma, MO) was measured in a similar assay, except that only one serum dilution (80-fold) was used, and the samples were spiked with the indicated amounts of inhibitors, or were diluted with an equivalent volume of PBS. To quantitate the amount of LEH-bound antibodies following incubation with undiluted serum, similar ELISA was carried out as above, except that LEH was first incubated with serum and was washed in PBS (three times) before being used to coat the wells of microtiter plates.

Human blood was obtained from healthy volunteers by a protocol approved by the Walter Reed Army Medical Center Human Use Committee. It was allowed to clot at room temperature and the serum was stored at −80°C before incubation with LEH (1 h at 37°C with shaking) at a LEH to serum volume ratio of 1:5 (20 mM LEH phospholipid). After incubation, vesicles were separated from the serum by centrifugation and hemolytic C activity was determined in the serum by the sheep red cell hemolysis assay, as described earlier [6].

Fig. 1 shows the titration curves for natural anti-LEH IgG and IgM antibodies in a volunteer's serum, as measured by ELISA. End-point dilution factors for IgG and IgM, defined as the highest serum dilution where A_{405} was still significantly higher than the

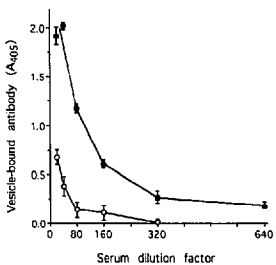

Fig. 1. Titration of LEH-reactive natural IgG (○) and IgM (●) antibodies in the serum of a healthy volunteer. Antibody binding to LEH was determined in solid phase ELISA, using αα Hb-containing LEH as antigen, as described in the Methods. Values are means ± S.E.M. for 4 or 5 wells, corrected for background (i.e. for A_{405} in non-coated wells).

background, were 320 and ≥ 640, respectively. Similar measurements in the sera of 8 volunteer individuals (including the one presented above) gave 80 as the median of end-point dilution factors for LEH-reactive IgG (range: 20 and 320, with no detectable antibody binding in 1 serum), and ≥ 640 for IgM in 8/8 sera. These data suggest that most human sera contain low titers of natural IgG and IgM antibodies that shows reactivity to LEH, with IgM titers exceeding those of IgG.

In order to clarify the specificity of anti-LEH antibodies, i.e. whether they are directed against the liposome membrane and/or against membrane-exposed Hb and/or HSA, we have utilized the fact that the binding of anti-phospholipid antibodies to liposomes can be inhibited with organic phosphates (ATP, phosphorylcholine chloride), but not by choline chloride [30]. The experiment testing the effect of the latter compounds on IgM binding to LEH revealed significant inhibition by 30 mM ATP and 10 mM phosphocholine, but not by 10 mM choline chloride (Fig. 2). Hence, a substantial portion of anti-LEH antibodies appeared to be phospholipid specific, with binding affinity to the phosphate groups on the phospholipid head groups of liposome bilayers [30]. This observation is in keeping with the fact that low levels of natural antibodies with phospholipid specificity are widespread in all animal species [31].

Concerning the role of anti-LEH antibodies in LEH-induced C activation, Fig. 3 shows a significant positive correlation between C consumption and the amount of LEH-bound IgM in 12 sera, which provides strong indication for a causal relationship between C activation and the binding of IgM to the vesicles. Such relationship was not observed between LEH-bound IgG and C consumption (data not shown), which difference could be explained by the lower efficiency of IgG to complex C1q in comparison with IgM.

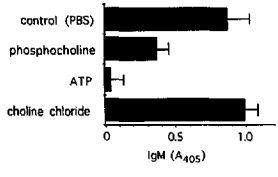

Fig. 2. The effect of organic phosphate compounds on the binding of IgM to LEH in the serum of a healthy volunteer. The effects of 10 mM phosphocholine, 30 mM ATP and 10 mM choline chloride on IgM binding to LEH was determined by ELISA, as in Fig. 1. Serum from a volunteer donor was diluted 80-fold with PBS and were spiked with the inhibitors or an equal volume of PBS (control). Bars are means ± S.E.M. for 4 or 5 wells, corrected for background.

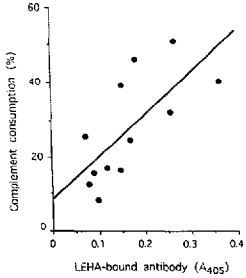

Fig. 3. Correlation between LEH-induced C consumption and the binding of IgM antibodies to LEH in 12 normal human sera. LEH-bound IgM was determined by solid phase ELISA, using serum-exposed $\alpha\alpha$Hb-containing LEH as antigen, as described. Background-corrected A_{405} values were plotted against C consumption, expressed as % decrease in CH_{50}/ml. Complement consumption was estimated by measuring hemolytic C activity in the serum, as described. Significant correlation at $P = 0.01$ ($r = 0.70$), as determined by regression analysis.

Consistent with a primary role of liposomal phospholipids in LEH-induced C activation, previous experiments [7,12] showed only minor quantitative differences between LEH and empty (PBS-containing) liposome-induced C activation. Also, control studies indicated that the procedures involved in the preparation of LEH (microfluidization, membrane filtration) are not responsible for the C activating potency of LEH (data not shown). Thus, beyond the implications regarding a potential adverse effect of LEH as a blood substitute, our results contribute to the understanding the interactions of liposomes with the C system. In particular, anionic liposomes have been shown earlier to activate C through the CP [16], while a recent study described that anionic liposome-induced C activation manifested itself at a lower sur-

face charge in the presence of immunoglobulins than in their absence [25]. These data together with the present study suggest that C activation by anionic liposomes can be induced by more than one mechanism. However, once IgM is involved, the efficiency of activation may be amplified, so that the antibody-mediated process prevails over other mechanisms.

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